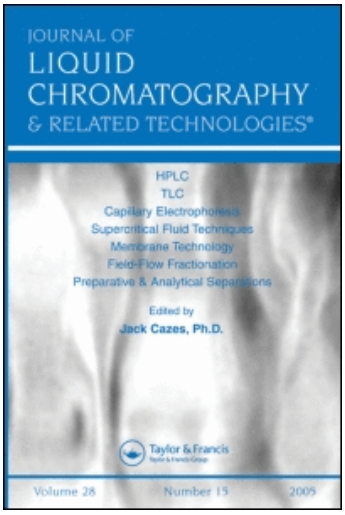

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 01 October 2000

To cite this Article Tanaka, Shunitz , Mori, Masanobu , Kodama, Kokoro and Hu, Wenzhi(2000) 'DETECTION OF BIOTIN BASED ON AN EXCHANGE REACTION WITH AVIDIN-HABA COMPLEX USING CAPILLARY ELECTROPHORESIS', Journal of Liquid Chromatography & Related Technologies, 23: 2, 197 — 207
To link to this Article: DOI: 10.1081/JLC-100101445
URL: http://dx.doi.org/10.1081/JLC-100101445

J. LIQ. CHROM. & REL. TECHNOL., 23(2), 197–207 (2000)

DETECTION OF BIOTIN BASED ON AN EXCHANGE REACTION WITH AVIDIN-HABA COMPLEX USING CAPILLARY ELECTROPHORESIS

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ABSTRACT

The detection of biotin at lower concentration in UV-visible region is difficult because of the low adsorption coefficients. In order to improve this problem, a new migration system, using an exchange reaction between biotin injected and avidin- HABA (2[4'-hydroxyazobenzene] benzoic acid) complex added in running buffer, was developed in the capillary electrophoresis (CE). The use of combination of zwitterionic surfactant (Zwittergent 3-14) and C8 pre-coating capillary enabled this migrating system by suppressing adsorption of avidin for a capillary wall effectively. In this migrating system, the reverse peak height of avidin-biotin complex increased with dependence on concentration of biotin injected. Moreover, the separation of biotin and biocytin was achieved as avidin complex at pH 7 of running buffer. The detection of biotin, contained in a vitamin tablet, was selectively detected avoiding other vitamins by using this CE system. This method enabled detection of only substances having strong affinity with avidin.

INTRODUCTION

Biotin is vitamin H, found in tissue or blood, and plays important roles in vivo as one of the biologically active molecule.[1] The roles contain metabolism of amino acid and synthesis of aliphatic acid.[2,3] Moreover, biotin binds strongly to avidin, which is a glycoprotein found in tissue or egg white.[4] The avidin-biotin interaction is known to be one of the strongest noncovalent interactions ($K_a=10^{15}$) between protein and ligand.[1] The avidin-biotin interaction has been used in various fields, for example, affinity purification,[5-6] protein, and nucleic acid blotting,[7] and enzyme-linked immunosorbent assay.[8] Therefore, to analyze biotin is important for biochemistry and clinical chemistry.

Many assay procedures[9-10] and affinity chromatography[11] for the determination of biotin have been developed. However, the sensitivity for biotin based on the measurement of UV-visible absorbance was not satisfied, because biotin has low adsorption coefficients at UV-visible region. Przyjazny et al.[12] used the avidin-biotin interaction in a post-column of HPLC in order to improve the sensitivity of biotin. They described the advantage of the change in the optical properties of the dye HABA (2-[4'-hydroxyazobenzene] benzoic acid) upon binding to avidin, and improved the selectivity and sensitivity of biotin and biocytin.

CE has recently become a powerful tool for the separation of biocomponents and the evaluation of bioaffinity interactions. We previously reported the separation of biotin and its relatives using capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC).[13] Separation of biotin relatives was achieved in SDS-MEKC, but the sensitivity for biotin was not enough, because biotin does not possess a strong chromophere. In this study, the introduction of avidin–biotin interaction into CE was investigated in order to improve the sensitivity of the detection and the selectivity of the migration of biotin. Recently, Okun et al.[14] and Vandernoot et al.[15] also investigated the uses of avidin-biotin interaction in CE. They used avidin complex pre-reacted out of a capillary but did not use the dynamic reaction in capillary column. We attempted to use an exchange reaction between biotin and HABA for avidin occurred in a capillary of CE. We also studied about the addition of zwitterionic surfactant (Zwittergent 3-14) into running buffer in order to suppress adsorption of avidin on the capillary wall.

EXPERIMENTAL

Reagents

Avidin from egg white was purchased from Wako Pure Chemical Inc. (Osaka, Japan). D-biotin (Vitamin H) and biocytin (Nε-biotinyl-$_L$-lysine) were purchased from Sigma Chemical Co. (St Louis, MO, USA). HABA (2[4'-

hydroxyazobenzene] benzoic acid) was purchased from Pierce (Rockford, Ill, USA). These samples were stocked as 4 mM solution with 0.1 M sodium hydroxide. N-tetradecyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14, $CH_3(CH_2)_{13}N(CH_3)_2SO_3Na$) was purchased from Calbiochem (La Jolla, CA, USA). Vitamin tablet containing biotin was prepared in a drugstore.

Apparatus and Condition of Migrating System

The untreated silica capillary (365 m O.D and 50 μm I.D.) use for CZE and MEKC was purchased from GL Sciences (Tokyo, Japan). The pre-coating capillary, CElect H 175 (C8 phase, 365 μm O.D and 75 μm I.D.) was purchased from SUPELCO (Bellefonte, PA, USA). Spectra phoresis 2000 (Thermo Separation Products, San Jose, CA, USA) was used to measure electropherograms. Both untreated and coated capillaries were operated at total length of 68.2 cm and effective length of 58.2 cm. The measurement of absorbance was performed at 500 nm, the adsorption maximum of avidin-HABA complex. Injection of the sample solutions was performed by a hydrodynamic method from the positive end for 15 s. The voltage was applied to 8-10 kV. 0.05% (v/v) benzyl alcohol was used as EOF marker. UV-visible spectra of avidin-biotin and avidin-HABA complex were performed at 400-600 nm by using photodiode array detection of Spectra phoresis 2000. The change of the spectra of avidin complex formed in test tubes was measured from 400 nm to 600 nm by U best V-550 spectrophotometer (JASCO, Tokyo, Japan).

In our CE system, a running buffer filled in the capillary was prepared as below. Sodium phosphate (10 mM NaH_2PO_4/Na_2HPO_4) with 10 mM Zwittergent 3-14 in water were run for 30 minutes in C8 coating capillary, and left for 30 min. And then sodium phosphate buffer (20mM) added to 7.5 μM avidin-37.5 μM HABA complex solution was migrated for 10 minutes. In MEKC for separation of a vitamin tablet, 20 mM sodium borate buffer with 50 mM SDS (pH 9) was used. All running buffers were adjusted to 50 mM ionic strength with 0.1M sodium chloride. After experiments, C8 coating capillary was washed with 50% (v/v) acetonitrile for 5 minutes and filled to 50% (v/v) acetonitrile. On the other hand, an untreated silica capillary was washed with methanol for 2 minutes, deionized and distilled water for 2 minutes, and then 0.1 M sodium hydroxide was filled.

Preparation of Sample Solution

The vitamin tablet contains riboflavin (vitamin B2), riboflavin sodium phosphate (vitamin B2 phosphate), pyridoxine hydrochloride (vitamin B6), thiamin nitrate (vitamin B1 nitrate), nicotinamide, and calcium (+)-pantothenate, besides biotin. This was prepared by a drugstore and the amount of biotin in the vitamin tablet was found to be 0.05mg/10 mg. The tablet (10 mg) was ground

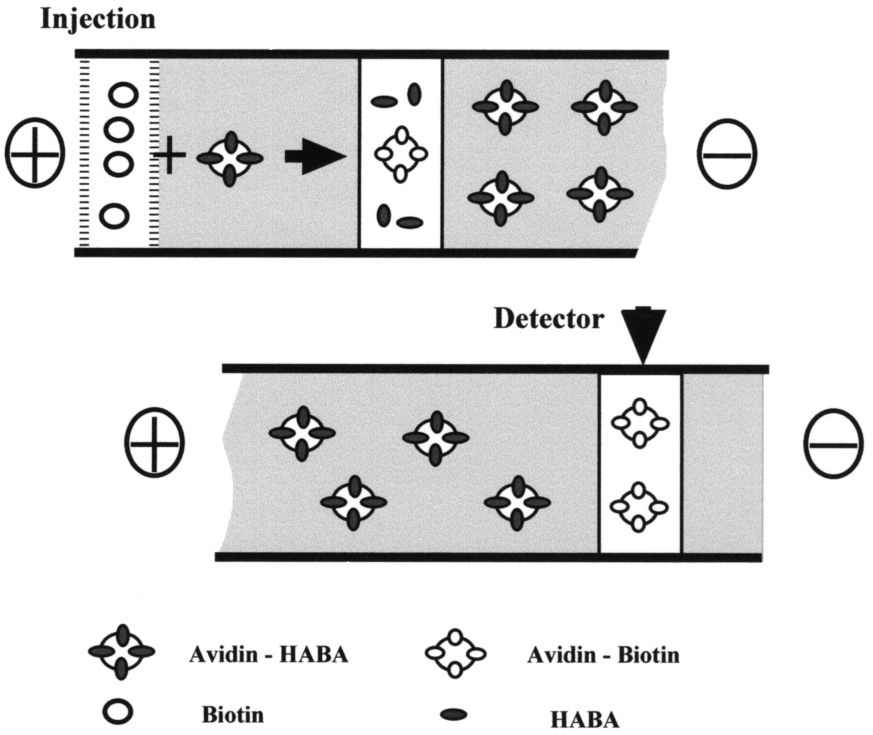

Figure 1. Schematic presentation of an exchange reaction between HABA and biotin to avidin obtained in capillary column. Top: Biotin is injected into running buffer with avidin-HABA complex at anode. Bottom: Avidin-biotin complex formed by exchange reaction is migrated to detector.

by mortar with a pestle, and biotin was extracted with 10 mL of 0.1 M sodium hydroxide, and then the solution, after filtration by 0.02 μm cellulose acetate membrane filter (Tokyo Roshi Kaisha, Ltd., Tokyo, Japan) was analyzed as a sample solution.

RESULTS AND DISCUSSION

The Improvement of Detection of Biotin Using an Exchange Reaction in CE

The detection of biotin at lower concentration in UV-visible range is difficult because of the low adsorption coefficients of biotin. In order to improve this problem, we attempted to introduce to the CE system a method

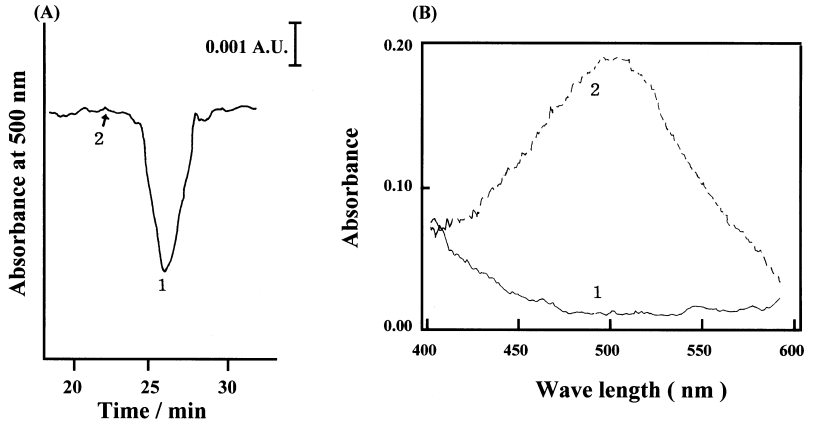

Figure 2. Change of spectra for avidin complex by photodiode array detection. (A) Electropherogram of avidin-biotin complex formed in capillary; Concentration of biotin: 30 μM; Buffer conditions: 7.5 μM avidin–37.5 μM HABA with 20 mM sodium phosphate (pH 7); Capillary column: CElect H175 (75 μm I.D.); Capillary length: 68.2 cm total and 58.2 cm to detector; Injection time: 10 s; Voltage: 10 kV; Detector; visible range at 500 nm. (B) UV-visible spectra at peak top and base line obtained by electropherogram; UV-visible absorbance region by photodiode array detection was performed from 400 nm to 600 nm.

using an exchange reaction in a post-column developed by Przyjazny et al.[12] Figure 1 shows a schematic presentation based on an exchange reaction for avidin complex. Avidin-HABA complex, whose adsorption maximum is at 500 nm,[16] is filled in the capillary column. An exchange reaction from avidin-HABA to avidin-biotin occurs in running buffer when biotin is injected, because the binding constant of avidin-biotin complex ($K_a=10^{15}$)[1] is much larger than that of avidin-HABA ($K_a=7\times10^6$).[16]

Avidin-biotin complex formed by an exchange reaction in capillary is electromigrated with the mobility of the complex. Since avidin-biotin complex does not have adsorption at 500 nm, the measurements at the wavelength will give a decreased peak of avidin-biotin. In this migrating system, it is important to suppress adsorption of avidin on a capillary wall. Avidin strongly absorbs on the capillary wall because of the positive charge of avidin. Previously, we found out that the combination of C8 coating capillary and Zwittergent 3-14 is effective to suppress adsorption of avidin and some proteins.[17] Therefore, in this system, C8 coating capillary adding Zwittergent 3-14 was used for this purpose. The EOF mobility in the CE system was approximately 1.8×10^{-4} cm^2/Vs at pH7, and less than that in the CE using an untreated silica capillary.

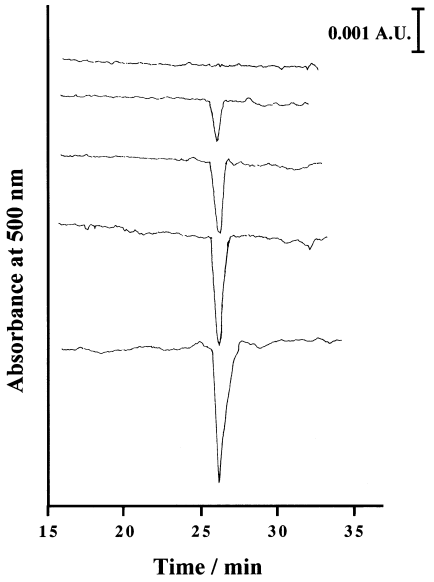

Figure 3. Electropherograms at various concentration of biotin injected. Concentrations of biotin injected: 0, 2.5, 5, 7.5, and 10 μM (in order from up to bottom); Injection time: 15 s; Voltage: 10 kV; Detector: visible range at 500 nm. Other conditions: same as in Figure 2.

Therefore, the avidin-biotin complex having positive charge will be migrated mainly by electrophoretic mobility. HABA liberated from avidin complex seems to be migrated to the reverse direction of avidin-biotin complex due to the negative charge in this pH region.[16]

Figure 2 shows an electropherogram of avidin-biotin complex formed by an exchange reaction. When biotin was injected into the running buffer containing avidin-HABA complex, biotin could be detected as decreasing of absorbance at 500 nm (Figure 2A). It shows that avidin-biotin complex was formed by an exchange reaction with avidin-HABA in a capillary. In order to confirm that, UV-visible spectra from 400 to 600 nm at peak top and at base line of the electropherogram by photodiode array detection were measured (Figure 2B). The UV-visible spectra at base line showed maximum value of adsorption at approximately 500 nm, the same as that of avidin-HABA complex. On the other hand, the spectra at peak top did not show such spectra. These results agreed with the results of reaction in test tube, and showed that an exchange reaction from avidin-HABA complex to avidin-biotin complex was practically performed in the capillary.

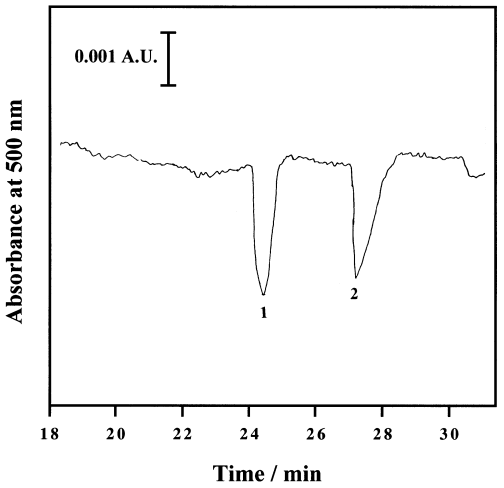

Figure 4. Separation of a mixture of biotin and biocytin using avidin affinity in CE. Sample numbers: (1), 7.5 μM biocytin, (2), 7.5 μM biotin; Injection time: 10 s; Voltage: 8 kV; Other conditions: same as Figure 2.

Figure 3 shows the electropherogram with increasing concentrations of biotin injected from 0 to 10 μM. In our previous study, normally using CZE and MEKC, it was difficult to detect 10 μM or below of biotin at UV detector. The system using an exchange reaction between avidin-HABA and avidin-biotin was useful enough to solve this problem, and then the peak height increased linearly depending on amounts of biotin injected. The peak shape was somewhat broad. It seems to be due to a nonspecific binding of biotin to avidin by electrostatic interaction. However, the detection limit of biotin in this system could typically expand more than that CE system.

Separation of a Mixture of Biotin and Biocytin

Figure 4 shows the electropherogram of a mixture of biotin and biocytin using an exchange reaction with avidin-HABA complex. Since biocytin also has a high affinity for avidin, it seems to be migrated as avidin-biocytin complex similarly to avidin-biotin complex. As shown in Figure 4, biocytin bound strongly to avidin was detected earlier than avidin-biotin complex. Avidin complexes are generally migrated as the species with positive charge at this pH.[18] However, biotin possessing a carboxyl group has a negative charge due to deprotonate at pH above 3.5.[3] On the other hand, biocytin is neutral in pH 7, due to possession both of an ε-amide group and a carboxyl group.[13]

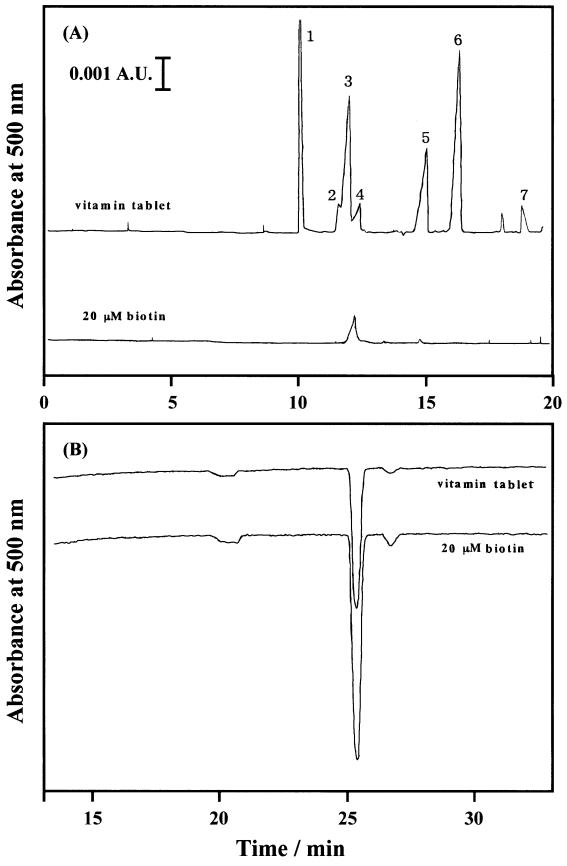

Figure 5. Detection of biotin in a vitamin tablet by MEKC and by CE using the exchange reaction. Sample; vitamin tablet containing 0.05 mg biotin was adjusted to 10 mg/10 mL with 0.1 M NaOH. 20 μM Biotin was used as standard. MEKC (A): 50 mM SDS with 20 mM sodium borate (pH9); Sample numbers: (1) nicotinamide, (2) pyridoxine hydrochloride, (3) pantothenate, (4) biotin, (5) riboflavin, (6) riboflavin phosphate, (7) thiamin nitrate; Capillary: untreated silica (50 μm I.D. 62.8 cm total and 52.8 cm to detector); Injection time: 15 s; Voltage: 15kV; Detector: UV at 210 nm. CE using avidin-biotin interaction (B): Same as in Figure 2.

Therefore, the difference of charge of functional group between them seems to be attributed to the difference of total charge of avidin-biotin and avidin-biocytin complex to lead to the separation of them. HABA liberated by an exchange reaction with biotin from avidin complex could not be detected in a voltage of direction from anode to cathode. However, when reverse voltage was applied, the HABA was detected as an increase peak in the absorbance at 348 nm,[16] as adsorp-

tion maximum of HABA. HABA was migrated as a negative charge at pH 7 in this CE system, and the electrophoretic velocity seemed to have migrated faster than EOF velocity. In this migration system, when the affinity of injected samples with avidin were lower than that of HABA, the samples could not be migrated as cationic avidin complex. Therefore, one of the advantages of this method seems that only ligand compound strongly binding with avidin can be selectively detected in this CE system.

Detection of Biotin in a Vitamin Tablet

Figure 5 shows electropherograms of a vitamin tablet containing 20 μM biotin by MEKC and the CE using an exchange reaction with avidin-HABA complex. In CZE mode with 20 mM borate at pH9, the peak of biotin was not clearly detected because other vitamins interfered. In MEKC mode using an anionic surfactant of SDS, the peak of biotin was obtained but not a complete isolated peak as shown in Figure 5A.

In the CE based on the exchange reaction with avidin-HABA, the peak of biotin contained in the vitamin tablet was detected as an isolated peak of avidin-biotin complex (Figure 5B), but the other vitamins were not detected. The peak (top in Figure 5B) was less than that of avidin-biotin complex pre-reacted under a static condition in the test tube (bottom in Figure 5B). It seemed to be due to the incomplete extraction of biotin from the vitamin tablet. However, the sensitivity of the peak was satisfied. Moreover, other negative charged vitamins not binding to avidin were not detected in this migrating system, because these were migrated into the direction of anode but not cathode.

The advantage of this CE is that only substances bound with avidin can be migrated and detected, therefore, other negative charged substances not bound to avidin will give no interference.

CONCLUSIONS

A new migration system using an exchange reaction between biotin injected and avidin-HABA complex added in running buffer was developed in the CE. This enabled detection of only substances having strong affinity with avidin, such as biotin and biocytin.

A separation of biotin and biocytin was achieved by different functional groups which interacted with avidin at pH 7. Moreover, the sensitivity for 20 μM or below concentration of biotin was improved much more than detection of biotin by typically CE mode. This system is applicable to effectively separate biotin relatives or biotinylated reagents in drugs, foods, and biomolecules.

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Received May 6, 1999
Accepted May 17, 1999
Manuscript 5057